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Isolation of a New Caryophyllane Ester from *Lactarius subumbonatus*: Conformational Analysis and Absolute Configuration

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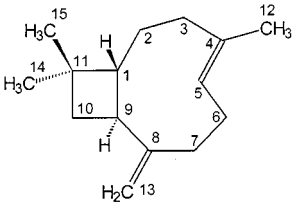
(1*R*,6*R*,9*S*)-6-Hydroxycaryophyllene (*S*)-6-hydroxystearate, a sesquiterpene alcohol esterified by the unusual fatty acid (*S*)-(+)-6-hydroxystearic acid, has been isolated from the fruiting bodies of the Basidiomycete *Lactarius subumbonatus*. Both NMR data and AM1 calculations indicate that the caryophyllene macrocyclic ring adopts a ββ conformation. The absolute configuration of the sesquiterpene moiety has been established by theoretical Circular Dichroism calculations (De Voe coupled oscillators theory) and that of the fatty acid by NMR studies of the (*R*)-2-methoxy-2-naphthylacetic acid (2-NMA) ester.

Introduction

Among the many sesquiterpene skeletons occurring in Nature (more than 90 different types are known[1]), β-caryophyllene derivatives are rather widespread and common in plants, but have been found much less frequently in higher fungi, namely in Basidiomycetes. The configuration of β-caryophyllene **1**, i.e. that bearing a *trans* fusion between the two rings, is the most common one in Nature. However, from fungi belonging to genus *Naematoloma* (Strophariaceae, Basidiomycetes) the naematolins, caryophyllene derivatives having the C-9 stereocenter epimerized (9-*epi*-caryophyllene), and therefore a *cis* junction between the two rings, have been isolated.[2][3] These compounds have also shown interesting biological activities, as cytotoxic and antiviral.

In the Russulaceae family (Basidiomycetes), the protoilludane skeleton and those biogenetically derived from it (i.e. marasmane, lactarane, secolactarane), are the most common sesquiterpene types.[4] On the other hand, only one example of caryophyllane skeleton has been reported so far,[5] namely 12-hydroxycaryophyllene-4,5-oxide, which has been isolated from the fruit bodies of *Lactarius camphoratus* (section Olentes[6]). This caryophyllene derivative shows a *trans* junction between the 4- and the 9-membered rings. From a biosynthetic point of view, both caryophyllanes and protoilludanes derive from a humulene precursor, though following two different cyclization patterns.[4]

During our screening of the secondary metabolites of Russulaceae as chemotaxonomic markers and potential pharmacological leads, we examined a still uninvestigated *Lactarius* species, viz. *L. subumbonatus* Lindgr. (synonym *L. serifluus* DC), belonging to section Olentes as *L. camphoratus*.[6] This mushroom is common in the broad-leaved woods of Mediterranean Italy, and is characterized by a strong, liquorice-like smell. Indeed, it has often been found[7] that the occurrence of peculiar organoleptic properties in the fruit bodies of several Basidiomycetes, as acrid or bitter tastes or strong smells, is related to the presence of secondary metabolites that are distinctive of the species or of the section.



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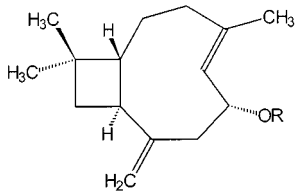

2a R = $CH_3(CH_2)_{11}CHOH(CH_2)_4CO$
2b R = H
2c R = PhCO

Results and Discussion

Intact fruit bodies of *L. subumbonatus* were minced at −20°C in CH_2Cl_2 (other specimens were extracted at room temperature for comparison, vide infra). This procedure has been demonstrated to be mild enough as to avoid the formation of chemical artifacts,[8] the low temperature being able to inactivate the enzymatic system of the mushroom, and CH_2Cl_2 being a particularly inert solvent. By this way, we readily established that only one sesquiterpene was present in intact fruit bodies, and this compound, **2a**, was easily purified on silica gel. From MS ($M^+ = 502$) and NMR spectra, it was identified as a sesquiterpene alcohol esterified by a fatty acid: this finding seems a general rule in Russulaceae, the only exception so far known being *Russula lepida*.[9] Hydrolysis of **2a** with NaOH in H_2O/EtOH afforded the corresponding sesquiterpene alcohol **2b** and free fatty acid **3a**.

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In the fruit bodies extracted at r.t. 5 min after grinding, **2a** is still the most abundant product. Small amounts of **2b** can be detected, however, meaning that the enzymatic system of the fungus slowly hydrolyzes the original sesquiterpene ester into the free alcohol and the fatty acid. When fruit bodies are ground at r.t., a more or less rapid enzymatic hydrolysis of the terpene esters contained in the intact cells has been reported for most Russulaceae species so far investigated.[4][7] For instance, in several acrid *Lactarius* or *Russula* species, the biological inactive stearoylvelutinal, contained in intact fruit bodies, is enzymatically converted into the pungent and toxic dialdehydes isovelleral or velleral as soon as the carpophores are injured: this mechanism is believed to constitute a chemical defence of the mushroom against parasites and predators.[10] In *L. subumbonatus* a similar behaviour is observed, given the lack of biological activity of ester **2a** compared to the activities of **2b** and **3a** (vide infra).

Structure and Absolute Configuration of the Sesquiterpene Moiety

The sesquiterpene alcohol **2b**, $M^+ = 220$ from EI-MS, showed in the ^{1}H-NMR spectrum, in addition to the alcoholic proton at $\delta = 4.60$, the presence of two exocyclic vinylic methylene protons at $\delta = 4.89$ and 5.00, one olefinic proton at $\delta = 5.26$, and one vinylic methyl group at $\delta = 1.62$, indicative of a β-caryophyllene structure. The *trans*-junction between the 4- and the 9-membered rings could be established comparing our NMR data with those reported in literature for other β-caryophyllene systems.[11] Notably, the resonances of the two quaternary methyl groups 14 and 15 in $CDCl_3$ are useful to this assignment: in fact, in *trans*-isomers they are found nearly isochronous in the proton spectrum, and well differentiated in the carbon one [in **2b** at $\delta = 0.93-0.97$ (^{1}H), and at $\delta = 22.6$ and 30.5 (^{13}C)]. From 2D-NMR (COSY, HMQC, HMBC), the position of the secondary alcoholic group was unambiguously located at C-6.

A sesquiterpene possessing the structure of 6-hydroxycaryophyllene has previously been isolated by Kaneko et al.[12][13] from extracts of *Betula pubescens*, and the ^{13}C-NMR spectrum reported in the literature is identical with that of **2b**. The two samples must therefore be identical, at least as their relative configurations are concerned, even if no stereochemical investigation could be afforded by the Japanese authors, owing to the limited amounts isolated (N. Kaneko, personal communication). Moreover, as no optical rotation was reported as well, it is impossible to state whether the product isolated from the plant and that isolated from the mushroom belong to the same antipodal series.

In order to assign the relative configuration of the C-6 stereocenter in **2b**, an analysis of the ^{1}H-NMR data, both ^3J-couplings and NOE correlations, was performed. The ^3J values are reported in Table 1 and the most significant NOEs observed are reported in Figure 1.

We promptly realized that a configurational assignment could not be done without a precise knowledge of the molecular conformation of 6-hydroxycaryophyllene. The conformational properties of β-caryophyllene **1** have been the subject of several theoretical and experimental investigations,[11,14−17] given the wide occurrence of **1** in Nature. From molecular mechanics calculations, four different conformations have been described, viz. ββ, βα, αα, αβ (Figure 2), which refer to the relative orientation of the two olefinic sections of the molecule.

In early studies,[11,14,15] an agreement was reached on the ββ and βα as being the two lowest-energy conformations, and as being almost degenerate. Contrary to these results, Fitjer et al. reported a detailed low-temperature ^{1}H- and ^{13}C-NMR study,[16] from which it appeared that the observed r.t. geometry was actually due to the overlap of two rapidly equilibrating conformers (plus a third one in slow exchange), but with the αα being the dominant conformation. This finding was also in agreement with an MM3 analysis of the same authors,[17] that indicated the αα as the most stable geometry of all.

In the light of the results obtained for the unsubstituted hydrocarbon **1**, we performed a theoretical conformational analysis on 6-hydroxycaryophyllene, to explore how the presence of the 6-OH group could affect the conformational properties of the caryophyllane skeleton. Moreover, a totally different calculation method was employed, i.e. we used the MO-SCF semiempirical AM1. Both relative configurations at C-6 were considered, and the set of the resulting eight different conformations of (1*R**,6*R**,9*S**)- and (1*R**,6*S**,9*S**)-6-hydroxycaryophyllene, is reported in Table 2.

For both stereoisomers AM1 calculated the ββ and βα as being the two lowest-energy conformations, while the αα and αβ were predicted to be significantly higher in energy. In particular, the βα conformation appeared to be the most stable one if the configuration at C-6 is (*S**), while the ββ was the most stable one when the configuration was (*R**). This was probably due to a severe steric interaction between the hydroxyl and the vinyl methyl groups, occurring in the βα conformation of the (6*R**) stereoisomer, and analogously in the ββ conformer of the (6*S**) stereoisomer. The relative populations of the various conformers at 298 K were computed after a Boltzmann analysis and are also reported in Table 2.

Both in the ^{1}H- and ^{13}C-NMR spectra of **2b**, a single set of resonances was detectable at 298 K, which was assigned to a single conformation, even if we did not perform any low-temperature experiment in order to ascertain the possible presence of fast conformational equilibria. In the NOESY spectrum (Figure 1), however, any significant contribution from the αα conformation could be ruled out, since no NOE was present either between H-5 and H-1, or between H-5 and H-7β, or any other which should be indicative of this geometry.[16] The interprotonic correlations expected for the αβ conformation were equally not detect-

Table 1. Experimental ^{1}H-NMR vicinal coupling constants (Hz) of **2b** compared with the calculated coupling constants of the different conformers of (1*R**,6*R**,9*S**)- and (1*R**,6*S**,9*S**)-hydroxycaryophyllene

	2b (exp)	(6*R**) (calcd)				(6*S**) (calcd)			
		ββ	βα	αβ	αα	ββ	βα	αβ	αα
$J_{1\beta,2\beta}$	< 1.0	1.0	1.3	1.0	1.0	1.1	1.3	1.3	1.0
$J_{1\beta,2\alpha}$	10.0	10.1	11.6	9.6	10.8	9.5	11.5	8.7	10.7
$J_{2\beta,3\beta}$	5.6	7.2	3.5	9.2	6.8	6.9	4.0	8.5	6.8
$J_{2\beta,3\alpha}$	< 1.0	0.6	3.0	0.3	0.8	0.8	2.5	0.3	0.8
$J_{2\alpha,3\beta}$	12.5	11.1	13.2	9.0	11.5	11.4	13.1	9.7	11.5
$J_{2\alpha,3\alpha}$	7.0	7.3	3.6	9.4	7.0	7.0	4.2	8.7	7.0
$J_{6,7\beta}$	6.8	6.3	7.1	1.8	1.5	2.4	1.8	11.6	11.6
$J_{6,7\alpha}$	9.8	10.0	9.2	4.7	5.1	3.8	4.8	3.2	2.6
$J_{9\alpha,1\beta}$	9.2	7.4	6.8	6.4	7.8	7.5	6.7	6.6	7.5
$J_{9\alpha,10\beta}$	9.0	7.1	6.7	6.4	7.6	7.1	6.6	6.5	7.3
$J_{9\alpha,10\alpha}$	8.5	10.2	10.3	10.3	10.1	10.2	10.3	10.3	10.1
$J_{5,6}$	10.4	11.3	6.1	7.6	6.1	3.6	7.6	6.0	10.7

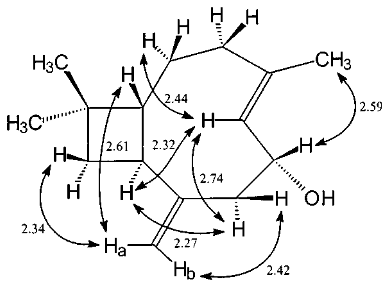


Figure 1. Significant NOESY correlations observed for **2b** and relative interprotonic distances [Å] as calculated by AM1 for the ββ conformation of (1*R**,6*R**,9*S**)-6-hydroxycaryophyllene

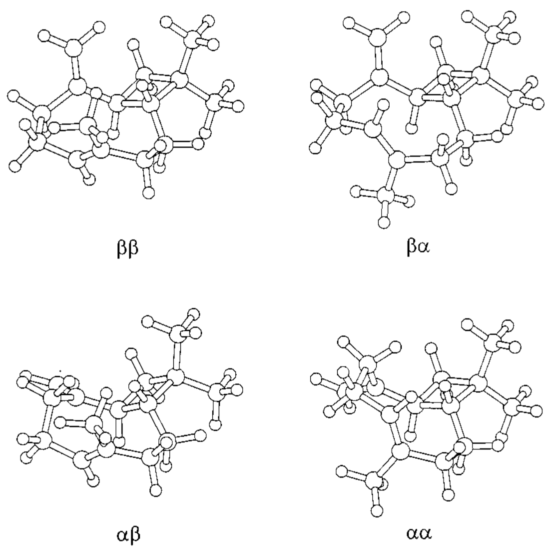
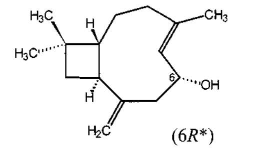
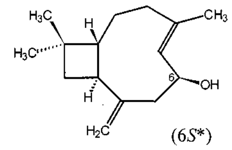


Figure 2. The four conformations of β-caryophyllene **1**

able. On the other hand, NOE was clearly visible between H-7β and H-13b, H-7α and H-9, H13a and H-10β, as expected from βα or ββ conformations. Even if the βα conformation of the (6*S**) stereoisomer and the ββ of the (6*R**) are locally almost plane symmetric, the ambiguity could be solved observing the characteristic NOEs between the olefinic H-5 and H-9, H-2α, and H-7α, which unequivocally indicated for **2b** a (6*R**) configuration and a ββ conformation. The vicinal *J*-coupling values calculated by the

Table 2. Heats of formation (kcal/mol), relative energies (kcal/mol), and populations (298 K) of the different conformers of (1*R**,6*R**, 9*S**)- and (1*R**,6*S**,9*S**)-6-hydroxycaryophyllene as determined by the AM1 program





	ΔH_f	E_{rel}	%	ΔH_f	E_{rel}	%
ββ	-38.69	0.00	86.0	-37.63	1.94	3.6
βα	-37.61	1.08	13.9	-39.57	0.00	96.3
αβ	-34.79	3.90	0.1	-32.94	6.63	<0.1
αα	-33.35	5.34	<0.1	-34.98	4.59	<0.1

Haasnot−Altona equation[18] for this geometry are also in better agreement with the experimental data. The observed ^{3}J values of **2b**, and those calculated for (6*R**)- and (6*S**)-6-hydroxycaryophyllene, are reported in Table 1.

In order to assess the absolute configuration, **2b** was converted into the corresponding benzoate **2c**. In fact, the "allylic benzoate" rule has often been used to establish the absolute configuration of allylic alcohols,[19] observing the sign of the benzoate CD band at about 230 nm. The mechanism giving rise to this CD band should be the nondegenerate exciton coupling between the long-axis polarised transition of the benzoate ring (at ca. 227 nm), and the $\pi-\pi^*$ transition of the olefinic chromophore (at ca. 195 nm); the helicity of the benzoate-olefin system determines the sign of the CD band.

Relative and even absolute configurations may also be established by X-ray crystallography. This would have been the more direct way to solve the problem, but compound **2b** did not give suitable crystals for X-ray analysis.

In the UV spectrum of **2c** two bands are present, one at 227 nm and another at 206 nm; in the CD spectrum (Figure 3), however, a broad negative band encompasses the 195−235 nm spectral region, with a minimum at 205 nm, while no distinct minimum or shoulder can be seen at 227 nm. The higher energy absorption band of the benzoate

chromophore has been object of both experimental[20][21] and theoretical investigations,[22] which seem to indicate that the strong, long-axis polarised transition observed at ca. 200 nm, is the result of two mutually orthogonal, almost degenerate transitions, the long-axis one being more intense. Anyway, these transitions have been very seldom used in absolute configuration assignments.

It should be stressed that in the application of "chirality rules", as the allylic benzoate one, care must be taken that no other chromophore is present in the molecule, absorbing in the same frequency range. Thus in β-caryophyllene structures, the presence of the second olefinic moiety (the exomethylene group) cannot be neglected.

In order to have a more quantitative interpretation of the CD spectrum of **2c**, we used a calculation method based on the De Voe coupled oscillators theory:[23] this method is suitable when the observed optical activity is dominated by an (electric) dipole − (electric) dipole coupling mechanism,[24] as it is reasonable to assume for our compound between 190 and 240 nm. Five oscillators were used to reproduce the electric-dipole allowed transitions of **2c** that can be considered the most significant ones in the investigated frequency range: one for each olefinic chromophore (195 nm, 5.0 Debye^2); two long-axis (227 nm, 12.0 D^2; 198 nm, 7.5 D^2) and one short-axis polarised (206 nm, 5.0 D^2) for the benzoate chromophore. An AM1 optimized geometry of **2c** was employed for the De Voe calculation: it turned out that the ββ conformation is again the lowest energy one.[25]

The experimental CD spectrum of **2c** is shown in Figure 3 along with the computed one for (1*R*,6*R*,9*S*)-6-hydroxycaryophyllene benzoate. Even if the negative maximum predicted by the calculation is about 5−6 nm blue-shifted compared with the experimental one, and the calculated bandwidth is narrower, the De Voe method provides the correct CD sign in the whole frequency range investigated. Moreover, the calculation predicts no distinct minimum at 227 nm, in agreement with experiment. This happens because the observed Cotton Effects of **2c** are dominated by the coupling between the two degenerate π−π* transitions of the olefinic chromophores: this figure gives the negative exciton couplet centered at 195 nm observed both in the experimental and in the calculated spectra (the former could be obtained up to ca. 190−191 nm). In contrast, the benzoate transitions give a smaller contribute, owing to the particular geometry of **2c**: we could see, by means of the De Voe program, that the benzoate long-axis transition at 227 nm gives a negative exciton couplet with olefin 4−5, and a similar, nearly mirror-like positive exciton with the exomethylene olefin, so that the two contributions to optical activity almost cancel out each other. An analogous situation is observed for the second long-axis transition and for the short-axis one of the benzoate chromophore (in the latter case a positive couplet is generated with olefin 4−5 and a negative one with olefin 8−13).

In conclusion, the absolute configuration of **2b** can be safely assigned as (1*R*,6*R*,9*S*), which corresponds to that previously established for other naturally occurring caryophyllenes.[11][26] So far it seems that only one enantiomeric series is present in Nature for this large family of sesquiterpenes.

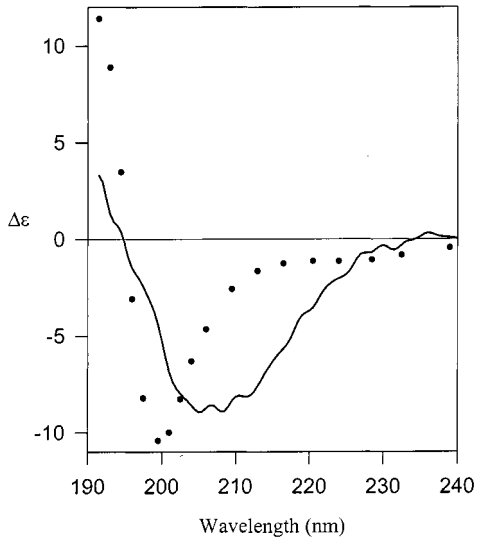
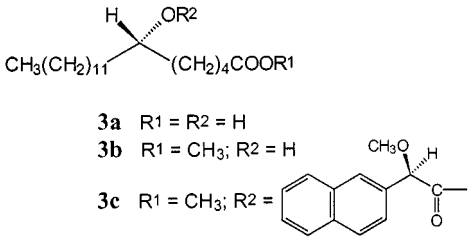


Figure 3. Experimental (continuous line) and theoretical (dots) CD spectra of **2c**

Structure and Absolute Configuration of the Fatty Acid Moiety

The carboxylic acid **3a** was converted into methyl ester **3b** by CH_2N_2 and was further investigated by NMR and GC-MS. The resonance at δ = 3.58 in the ^{1}H spectrum, correlated in the HETCOR spectrum with a ^{13}C methine resonance at δ = 71.9, showed that a secondary alcoholic function was present. Although the molecular peak at *m/z* 314 was not visible in the GC-MS spectrum, the presence of a peak at *m/z* 296, corresponding to $(M - H_2O)^+$, along with NMR data, allowed us to establish that **3a** was a hydroxystearic acid, while a prominent peak at *m/z* 145, corresponding to the loss of $C_{12}H_{25}$ from the molecular ion, suggested that the alcoholic group was at C-6. This is an interesting finding, since several *Lactarius* species contain the corresponding oxidized 6-ketostearic acid (lactarinic acid), similarly esterified with a sesquiterpene alcohol.[4] To confirm the structure of **3a**, a sample of 6-ketostearic acid previously isolated from *L. scrobiculatus*[27] was methylated with CH_2N_2 and then reduced with $NaBH_4$. The racemic compound thus obtained was analyzed by GC-MS, and both the retention time and the MS spectrum were identical to those of **3b**. The ^{13}C-NMR spectra were also identical.

Finally, to assign the absolute configuration of **3b**, we employed the recently introduced 2-methoxy-2-naphthylacetic acid (2-NMA) as NMR chiral shift reagent.[28][29] In the ^{1}H-NMR spectra of 2-NMA esters, larger and longer-range shielding effects are usually observed, when compared to those obtained with more traditional Mosher's methoxy trifluorophenylacetic acid (MTPA) or Trost's methoxy phenylacetic acid (MPA) esters. In this way the absolute configuration of nearly symmetrically substituted



secondary alcohols as **3b** can be determined even using a single enantiomer of the acid.[28]

The (*R*)-2-NMA ester (**3c**) was prepared and the ^{1}H- and COSY NMR spectra were recorded. In this way it could be assessed that the resonances of the protons belonging to the C7−C18 part of **3c** are strongly moved upfield when compared to the corresponding ones of the starting alcohol **3b** (δ_{H8} = 0.82 in **3c** vs. 1.42 in **3b**), while those of the C1′−C5 part are much less affected (δ_{H2} = 2.21 in **3c** vs. 2.32 in **3b**). Such chemical shift differences can be explained by the anisotropic diamagnetic shielding of the naphthalene ring on nearby protons: following the original model,[30] this means that the C7−C18 part of the alcohol and the aromatic ring of the acid are on the same side of a plane containing the carbinyl proton, the ester carbonyl group, and the methoxy group. In this way a configurational assignment can be done: for **3b** it leads to the (*S*) absolute configuration.

The geometry assumed by the Trost−Mosher′s method corresponds to a *syn*-periplanar (*sp*) conformation: anyway the latter is always in equilibrium with a second conformation, the *anti*-periplanar (*ap*) one, where the screening situation is reversed[31] (Figure 4). In order for the model to hold, one must assume that the *sp* conformation is the lowest-energy one independently of the alcoholic moiety. It has been recently reported, however, that this is not always the case,[32] and the application of Trost−Mosher′s model may thus lead to wrong assignments. A theoretical conformational analysis is therefore recommendable.

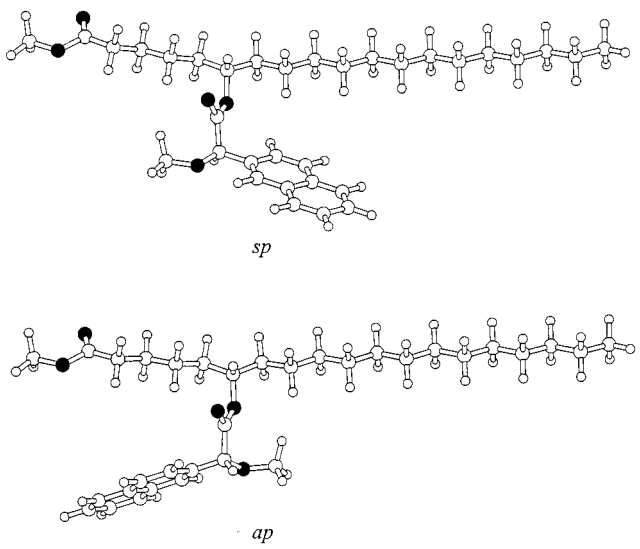


Figure 4. Examples of *sp* and *ap* conformations of compound **3c**

To this purpose, an AM1 study of the diastereomeric esters of (*R*)-2-NMA with methyl (*R*)- and (*S*)-6-hydroxystearate was performed. Actually, it turned out that for the two stereoisomers both the *sp* and the *ap* geometries are represented by families of a very large number of conformers, due to the many degrees of freedom of the system, making the exploration of the conformational space not so straightforward. Anyhow, if a (6*S*) configuration is assumed for **3b**, a certain bias towards an *sp* conformation was found, accordingly with Trost−Mosher′s model, but the energy difference with *ap* conformations was not large enough to support our assignment on a calculation ground. However, the absolute configuration of **3b** can be compared with that of a model hydroxy fatty acid ester, viz. methyl (*S*)-9-hydroxysterarate, for which the ^{1}H-NMR spectra of the (*R*)- and (*S*)-2-NMA esters leads to the correct configuration, after application of the Trost−Mosher′s model.[28]

A further interesting experiment has been proposed by Riguera et al.[33] to confirm configurational assignments by use of methoxy-arylacetic acids. According to these authors, since a decrease of the temperature leads to a population increase of the *sp* conformer, one should observe an increased screening of the protons belonging to one substituent (for **3b** H7−H18), and a decreased screening of the protons belonging to the other substituent (for **3b** H1′−H5). In the event, when the temperature of a $CDCl_3$ solution of **3c** was decreased from 298 K to 266 K, we observed that δ_{H8} moved from 0.82 to 0.74, while δ_{H2} moved from 2.21 to 2.24, in agreement with theory.

To our knowledge (*S*)-6-hydroxystearic acid, as well as the ester **2a**, have never been reported previously as natural products. The ester **2a** was found to be odourless, and therefore is not responsible for the strong smell of *L. subumbonatus* fruit bodies, that is still present in the raw extract.

Activity Assays

Toxicity against *Artemia salina*[34] was tested for caryophyllene ester **2a**, 6-hydroxycaryophyllene **2b**, and 6-hydroxystearic acid **3a**. While **2a** showed almost no activity, even at 100 ppm, **2b** had an LD_{50} of 11 ppm, and **3a** an LD_{50} of 28 ppm.

Experimental Section

Extraction and Isolation: Fruit bodies of *Lactarius subumbonatus* were collected in two different sites in the province of Rome (Italy), in December 1997 and in November 1998. Their contents of terpene metabolites were identical. A voucher specimen is available at the University of Pavia from the authors. Immediately after the collection, the fruit bodies were first frozen at −20°C and then extracted with CH_2Cl_2. The raw extract was dried over $MgSO_4$, filtered, and the solvent was evaporated in vacuo. From 250 g of fruit bodies, about 300 mg of raw extract could be obtained in this way. Flash chromatography of the crude extract was performed using silica gel pre-treated with NEt_3, to avoid decompositions on acidic sites. EtOAc/hexane mixtures were employed as eluents. Only freshly distilled solvents were used.

Materials and Methods: Melting points were determined on a Fisher-Johns hot-plate and are uncorrected. Optical rotations were determined on a Perkin−Elmer 241 polarimeter at 20°C. − FT-IR spectroscopy was performed on a Perkin−Elmer Paragon 1000 spectrometer. − UV spectra were recorded on a Perkin−Elmer Lambda 5 instrument, and CD spectra on a Jasco J-710 spectropolarimeter, employing 0.1 cm optical path cuvettes. − NMR spectra were recorded either on a Bruker 400 MHz or on a Bruker 600 MHz spectrometers. TMS was always used as internal standard. The mixing time for the NOESY experiment was set at 0.670 sec. − MS spectra were recorded on a Finnigan MAT 8222 spectrometer, operating at 70 eV; GC-MS was performed with a capillary GC Hewlett−Packard 5890 instrument, equipped with a mass selective detector HP 5970.

Methods of Calculation: Theoretical calculations were performed with the AM1 semiempirical method[35] and were carried out at the RHF level. The geometry of all the compounds investigated was fully optimized and energy minimized. Several optimizations from different starting geometries were performed to take into account all the possible conformations of each compound.

(1*R*,6*R*,9*S*)-6-Hydroxycaryophyllenyl (*S*)-6-Hydroxystearate (2a) was obtained as a whitish oil, $[\alpha]_D = -23$ ($c = 0.2$, $CHCl_3$) UV (Hexane) λ_{max} (ε): 214 nm (3700) IR (thin film): 3442, 3071, 2925, 2853, 1733, 1637, 1455, 1371, 1259, 1171, 1150, 1096, 1031, 971. − EI-MS: 502 [M^+] (<1), 433 (<1), 411 (<1), 333 (<1), 283 (100), 265 (39), 247 (11), 220 (18), 203 (48), 202 (35), 147 (31), 133 (53), 69 (71), 55 (71). − ^{1}H NMR (C_6D_6): 5.91 (1 H, ddd, H-6, J = 10.4, 10.2, 6.5) 5.38 (1 H, d, H-5, J = 10.2), 5.04 (1 H, s, H-13a), 4.95 (1 H, s, H-13b), 3.43 (1 H, br quintet, H-6′), 3.00 (1 H, dd, H-7β, J = 11.1, 6.5), 2.48 (1 H, m, H-3α), 2.29 (2 H, t, H-2′, J = 7.3), 2.20 (1 H, ddd, H-9, J = 9.8, 8.4), 2.15 (1 H, dd, H-7α, J = 11.1, 10.4), 1.82 (1 H, dd, H-10α, J = 10.7, 8.4), 1.75 (3 H, s, H-12), 1.67 (2 H, m, H-3′), 1.65 (1 H, dd, H-10β, J = 10.7, 9.8), 1.52−1.44 (4 H, m, H-1, H-2α, H-2β, H-3β) 1.42−1.30 (ca. 26 H, br m, H-4′-5′ and H-7′-17′), 0.99 (3 H, t, H-18′), 0.97 (3 H, s, H-14 or 15), 0.93 (3 H, s, H-15 or 14). − ^{13}C NMR (C_6D_6): 173.3 (s, C-1′), 150.3 (s, C-8), 139.0 (s, C-4), 125.7 (d, C-5), 114.2 (t, C-13), 73.3 (d, C-6), 72.0 (d, C-6′), 56.7 (d, C-1), 49.8 (d, C-9), 46.6 (t, C-7), 43.1 (t, C-10), 38.7 (t), 38.3 (t), 35.7 (t, C-3), 35.4 (t, C-2′), 33.4 (s, C-11), 33.0 (t), 32.0 (t), 30.9 (t), 30.8 (t), 30.5 (t), 30.5 (q, C-14 or C-15), 26.8 (t) 26.2 (t), 26.0 (t), 23.8 (t), 23.6 (q, C-12), 22.6 (q, C-15 or C-14), 15.0 (q, C-18′).

Hydrolysis of 2a: 20 mg of **2a** were dissolved in 96% EtOH. About 0.5 mL of 4 N NaOH were added and the solution was warmed at 50°C for 15 min; after this time the reaction was complete. Following removal of EtOH in vacuo, the solution was extracted with CH_2Cl_2 and the organic phase was dried with $MgSO_4$, to yield about 7 mg of **2b**. The aqueous layer was acidified with 6 N HCl and extracted with EtOAc. After drying with $MgSO_4$, 10 mg of the acid **3a** were obtained. **3a** was then added with an Et_2O solution of CH_2N_2 to yield methyl ester **3b** in a quantitative fashion.

(1*R*,6*R*,9*S*)-6-Hydroxycaryophyllene 2b was obtained as colourless needles, m.p. 46−49°C, $[\alpha]_D = -51$ ($c = 0.4$, $CHCl_3$). − IR (thin film): 3323, 3069, 2953, 2855, 1636, 1451, 1380, 1085, 1025, 888. − EI-MS: 220 [M^+] (5), 205 (10), 202 (8), 187 (12), 177 (18), 149 (24), 137 (28), 123 (35), 121 (48), 109 (88), 107 (44), 95 (69), 81 (90), 69 (92), 41 (100). − ^{1}H NMR ($CDCl_3$): 5.26 (1 H, dt, C-5, J_{5-6} = 10.4, J_{5-12} = 1.4), 5.00 (1 H, s, H-13a), 4.89 (1 H, s, H-13b), 4.60 (1 H, ddd, H-6, $J_{6-7\alpha}$ = 9.8, $J_{6-7\beta}$ = 6.8), 2.78 (1 H, dd, H-7β, $J_{7\alpha\text{-}7\beta}$ = 11.0), 2.53 (1 H, br dd, H-3α, $J_{2\alpha\text{-}3\alpha}$ = 7.0, $J_{2\beta\text{-}3\alpha}$ < 1.0), 2.27 (1 H, dd, H-9, $J_{1\beta-9\alpha}$ = 9.2, $J_{9\alpha-10\alpha}$ = 8.5, $J_{9\alpha-10\beta}$ = 9.0), 1.94 (1 H, dd, H-7α), 1.78 (1 H, dd, H-10α, $J_{10\alpha\text{-}10\beta}$ = 10.7), 1.67 (1 H, m, H-2α, $J_{1\beta-2\alpha}$ = 10.0, $J_{2\alpha\text{-}3\beta}$ = 12.5), 1.62 (3 H, s, H-12), 1.60 (2 H, m, H-10β and H-2β, $J_{1\beta\text{-}2\beta}$ < 1.0, $J_{2\beta\text{-}3\beta}$ = 5.6), 1.53 (1 H, m, H-3β), 1.42 (1 H, dd, H-1), 0.97 (3 H, s, H-14 or 15), 0.96 (3 H, s, H-15 or 14). − ^{13}C NMR: 150.2 (s, C-8), 137.1 (s, C-4), 128.0 (d, C-5), 112.6 (t, C-13), 70.9 (d, C-6), 55.7 (d, C-1), 49.4 (t, C-7), 49.1 (d, C-9), 42.4 (t, C-10), 34.9 (t, C-3), 32.9 (s, C-11), 31.4 (t, C-2), 30.0 (q, C-14 or C-15), 23.1 (q, C-12), 22.0 (q, C-15 or C-14).

Preparation of (1*R*,6*R*,9*S*)-6-hydroxycaryophyllenyl Benzoate 2c: To a solution of 3.5 mg of **2b** in pyridine, an excess of benzoyl chloride and a catalytic amount of DMAP were added at 30°C. After 2 h the reaction was quenched with a diluted $NaHCO_3$ solution. Excess pyridine was eliminated washing with aqueous $CuSO_4$. After extraction with *n*-hexane and purification on silica-gel, **2c** was obtained as a whitish oil (1.5 mg), MS (EI): 324 [M^+] (1), 309 (<1), 202 (17), 133 (35), 105 (100), 77 (55).− UV (Hexane) λ_{max} (ε): 227 nm (6000), 206 nm (4900). − CD (Hexane) λ_{max} (Δε): 252 nm (−0.23), 205 nm (−8.6). − ^{1}H NMR ($CDCl_3$): 8.04 (2 H, d, H-1′), 7.55 (1 H, t, H-3′), 7.43 (2 H, t, H-2′), 5.75 (1 H, ddd, H-6), 5.38 (1 H, d, H-5), 5.09 (1 H, s, H-13a), 5.03 (1 H, s, H-13b), 2.93 (1 H, dd, H-7β), 2.58 (1 H, br m, H-3α), 2.33 (1 H, dd, H-9), 2.16 (1 H, dd, H-7α), 1.81 (1 H, dd, H-10α), 1.76 (3 H, s, H-12), 1.69 (1 H, m, H-2α), 1.61 (3 H, m, H-10β, H-2β and H-3β), 1.46 (1 H, t, H-1), 0.98 (3 H, s, H-14 or 15), 0.97 (3 H, s, H-15 or 14).

Methyl (*S*)-6-Hydroxystearate 3b was obtained as white crystals, m.p. 43−44°C, $[\alpha]_D = +2$ ($c = 0.4$, MeOH). − GC-MS: 296 [$M - H_2O^+$] (1), 264 (2), 222 (1), 180 (1), 145 (52), 127 (3), 116 (50), 113 (68), 95 (10), 87 (100), 67 (40), 57 (45), 55 (68). − ^{1}H NMR ($CDCl_3$): 3.66 (3 H, s, H-1′), 3.58 (1 H, m, H-6), 2.32 (2 H, t, H-2), 1.63 (m), 1.42 (m), 1.25 (br), 0.87 (3 H, t, H-18). − ^{13}C NMR: 174.3 (s, C-1), 71.9 (d, C-6), 51.7 (q, C-1′), 37.7 (t), 37.2 (t), 34.2 (t), 32.1 (t), 29.8 (t), 29.5 (t), 25.8 (t), 25.4 (t), 25.1 (t), 22.9 (t), 14.3 (q, C-18).

Preparation of the (*R*)-2-NMA Ester 3c: 2-NMA was synthesized and resolved as described in the literature.[28] **3c** was prepared reacting (*R*)-2-NMA chloride (15 mg of (*R*)-2-NMA plus a 10-fold excess of oxalyl chloride in CH_2Cl_2, 3 h, r.t.) with 10 mg of **3b** in pyridine at 35°C for 2 h (a catalytic amount of DMAP was added). After silica gel purification of the reaction mixture, 3.2 mg of the ester was obtained. − MS (EI): 512 [M^+] (11), 297 (21), 172 (79), 171 (100). − ^{1}H NMR ($CDCl_3$): 7.87 (4 H, m), 7.49 (3 H, m), 4.92 (1 H, s), 4.91 (1 H, m), 3.65 (3 H, s), 3.47 (3 H, s), 2.21 (2 H, t), and a series of multiplets at δ = 1.58, 1.53, 1.34, 1.29, 1.25, 1.15, 1.07, 0.98, 0.91, 0.89, 0.82.

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[1] J. D. Connolly, R. A. Hill, *Dictionary of Terpenoids* Vol. 1, Chapman & Hall, London **1991**.
[2] Y. Ito, H. Kurita, T. Yamaguchi, M. Sato, T. Okuda, *Chem. Pharm. Bull.* **1967**, 15, 2009−2010.
[3] K. Doi, T. Shibata, M. Nara, S. Tsuboyama, T. Sakurai, K. Tsuboyama, *Chem. Lett.* **1986**, 653−656.

[4] G. Vidari, P. Vita-Finzi, *Sesquiterpenes and Other Secondary Metabolites of Genus Lactarius (Basidiomycetes): Chemistry and Biological Activity*. In: *Studies in Natural Product Chemistry*. Atta-ur-Rahman Ed., Vol. 17, p.153−206. Elsevier, Amsterdam, **1995**.
[5] W. M. Daniewski, P. A. Grieco, J. C. Huffman, A. Rymkiewicz, A. Wawrzun, *Phytochemistry* **1981**, *20*, 2733−2734.
[6] M. Bon, *Docum. Mycol.* **1980**, Tome X, Fascicule n. 40.
[7] O. Sterner, H. Anke, *Czech Mycol.* **1995**, *48*, 39−52.
[8] M. Clericuzio, F. Han, F. Pan, Z. Pang, O. Sterner, *Acta Chem. Scand.* **1998**, *52*, 1333−1337.
[9] G. Vidari, Z. Che, L. Garlaschelli, *Tetrahedron Lett.* **1998**, *39*, 6073−6076.
[10] O. Sterner, R. Bergman, J. Kihlberg, B. Wickberg, *J. Nat. Prod.* **1985**, *48*, 279−288.
[11] S. F. R. Hinkley, N. B. Perry, R. T. Weavers, *Phytochemistry* **1994**, *35*, 1489−1494.
[12] N. Kaneko, A. Sato, H. Ishii, T. Kanisawa, *Aroma components in the bud oil of Betula species*. In: *Proc. 12*th Int. Congress of Flavours, Fragrances and Essential Oils Vienna, Austria, **1992**.
[13] N. Kaneko, H. Ishii, I. Morimoto, S. Watanabe, *Jpn. Kokai Tokkyo Koho*, Pat. JP03 56,410.
[14] H. Shirahama, E. Osawa, B. R. Chabra, T. Shimokawa, T. Yokono, T. Kanaiwa, T. Amiya, T. Matsumoto, *Tetrahedron Lett.* **1981**, *22*, 1527−1528.
[15] G. Guella, G. Chiasera, I. N′Diaye, F. Pietra, *Helv. Chim. Acta* **1994**, *77*, 1203−1221.
[16] M. Hubner, B. Rissom, L. Fitjer, *Helv. Chim. Acta* **1997**, *80*, 1972−1982
[17] L. Fitjer, A. Malich, C. Paschke, S. Kluge, R. Gerke, B. Rissom, J. Weiser, M. Noltemeyer, *J. Am. Chem. Soc.* **1995**, *117*, 9180−9189.
[18] C. A. G. Haasnot, F. A. A. de Leeuw, C. Altona, *Tetrahedron* **1980**, *36*, 2783−2792.
[19] N. Harada, K. Nakanishi, *Circular Dichroic Spectroscopy*. Oxford Univ. Press UK **1983** and references therein.
[20] J. Tanaka, *Bull. Chem. Soc. Jpn.* **1963**, *36*, 833−847.
[21] A. Yogev, L. Margulies, Y. Mazur, *J. Am. Chem. Soc.* **1971**, *93*, 249−251.
[22] J. Catalàn, A. Macìas, *Bull. Soc. Chim. Belg.* **1976**, *85*, 1013−1016.
[23] H. De Voe, *J. Chem. Phys.* **1964**, *41*, 393−400. *J. Chem. Phys.* **1965**, *43*, 3199−3208.
[24] C. Rosini, M. Zandomeneghi, P. Salvadori, *Tetrahedron: Asymmetry* **1993**, *4*, 545−554.
[25] Two almost degenerate ββ minimum energy conformations are found by AM1 for benzoate **2c**, only differing in the tilt of the aromatic ring. Their calculated CD spectra are very similar, and the average of the two is reported in Figure 3.
[26] W. Klyne, J. Buckingam, *Atlas of Stereochemistry* Vol. 2. Chapman & Hall, London **1978**.
[27] M. De Bernardi, L. Garlaschelli, L. Toma, G. Vidari, P. Vita-Finzi, *Tetrahedron* **1993**, *49*, 1489−1504.
[28] T. Kusumi, H. Takahashi, P. Xu, T. Fukushima,Y. Asakawa, T. Hashimoto, Y. Kan, Y. Inouye, *Tetrahedron Lett.* **1994**, *35*, 4397−4400.
[29] J. M. Seco, S. Latypov, E. Quiñoà, R. Riguera, *Tetrahedron Lett.* **1994**, *35*, 2921−2924.
[30] B. M. Trost, J. L. Belletire, S. Goldeski, P. G. McDougal, J. M. Balkovec, J. J. Baldwin, M. E. Christy, G. S. Ponticello, S. L. Varga, J. P. Springer, *J. Org. Chem.* **1986**, *51*, 2370−2374 and references therein.
[31] J. M. Seco, S. Latypov, E. Quiñoà, R. Riguera, *Tetrahedron: Asymmetry* **1995**, *6*, 107−110.
[32] X. Shi, A. B. Attygalle, A. Liwo, M. H. Hao, J. Meinwald, H. R. W. Dharmaratne, W. M. A. P. Wanigasekera, *J. Org. Chem.* **1998**, *63*, 1233−1238.
[33] S. Latypov, S.; J. M. Seco, J. M.; E. Quiñoà, R. Riguera, *J. Am. Chem. Soc.* **1998**, *120*, 877−882.
[34] B. N. Meyer, N. R. Ferrigni, J. E. Putnam, L. B. Jacobsen, D. E. Nichols, J. L. McLaughlin, *Planta Med.* **1982**, *45*, 31−34.
[35] M. J. S. Dewar, E. G. Zoebisch, E. F. Healy, J. J. P. Stewart, *J. Am. Chem. Soc.* **1985**, *107*, 3902−3209.

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